# What confers specificity on glycine for its receptor site?

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1   The structural requirements for activation of the glycine receptor were studied in isolated ventromedial hypothalamic neurones of rats by use of a 'concentration-clamp' technique under single-electrode voltage-clamp conditions.

2   α-Amino acids (L-α-alanine, and D-α-alanine, and L-serine), and glycine-methylester, glycine-ethylester and β-amino acids (β-alanine and taurine) produced a transient inward $Cl^-$ current, which was similar to that induced by glycine.

3   The responses to individual α- and β-amino acids were selectively antagonized by strychnine, but were not affected by bicuculline, picrotoxin or the taurine antagonist, TAG (6-aminomethyl-3-methyl-4H,1,2,4-benzothiadiazine-1,1-dioxide hydrochloride), suggesting that α- and β-amino acids activate the same glycine receptor.

4   β-Amino acids were slightly more potent than the α-amino acids in causing cross-desensitization of the glycine response.

5   From the results of the structure-activity analysis of the optical isomers of α-alanine, serine and cysteine, a tentative structure of the glycine receptor is proposed.

## Introduction

Glycine plays an important role as an inhibitory neurotransmitter in the central nervous systems of various animals (Curtis *et al.*, 1968; Krnjević, 1974; Aprison *et al.*, 1976; Pycock & Kerwin, 1981). In spite of its simple chemical structure, many kinds of glycine binding sites have been reported in vertebrate tissues: (a) a conventional glycine receptor which operates through a $Cl^-$ channel and which is sensitive to strychnine (Curtis *et al.*, 1968; Graham *et al.*, 1985), (b) a strychnine-insensitive glycine binding site (De Feudis, 1977; Bristow *et al.*, 1986), one of which may allosterically regulate the N-methyl-D-aspartate (NMDA) receptor (Johnson & Ascher, 1987), and (c) a glycine transport carrier (Johnson & Iversen, 1971; Wilkin *et al.*, 1981). In general, for compounds with complex chemical structures and flexibility in the molecule, there exists the possibility of activity at different recognition sites, due to their conformational variation. It is, for example, generally accepted that GABA can exist in two distinct conformations: an extended or a folded form. GABA is believed to interact with its receptor in an extended conformation, while binding to the transport carrier protein requires the folded form

(Krogsgaard-Larsen *et al.*, 1975; Johnston *et al.*, 1976). Similarly, the ability of acetylcholine to exist in different conformational states, accounts for its ability to activate either nicotinic or muscarinic receptors. However, the interactions of glycine with its receptor sites are unlikely to be explained in this way, since glycine seems to exist only in one conformation. It is thus important to know what confers specificity upon the interaction between glycine and its receptor-ion channel complex in mammalian CNS neurones. Although previous electrophysiological studies have provided considerable information about the specificity of the glycine receptor, quantitative data have been lacking, because conventional methods such as bath perfusion, ionophoretic and pressure applications of glycine, were accompanied by significant desensitization before glycine equilibrated with the receptor.

Recently, we have developed a new type of concentration-jump technique termed 'concentration-clamp' which combines internal perfusion and very rapid drug application. This technique makes it possible to apply drugs within a few milliseconds (Akaike *et al.*, 1986). This high application speed greatly decreased or abolished the contamination of the activation phases with desensitization. In the present study, we have applied this 'concentration-clamp' technique to isolated ventromedial hypothalamic neurones of the rat (Kaneda *et al.*, 1988)

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and examined the effects of α- and β-amino acids and glycine derivatives on the glycine receptor. From this structure-activity analysis of glycine receptor interaction, the tentative structure of the glycine receptor is proposed.

## Methods

### Preparation

Experiments were performed on ventromedial hypothalamic (VMH) neurones isolated from 1 to 3 week-old Wistar rats by use of a previously described dissociation procedure (Kaneda et al., 1988).

### Solutions

The ionic compositions of the external and internal solutions were (in mM): (A) incubation solution for the enzyme treatment and isolation of cells: NaCl 160, KCl 5, $CaCl_2$ 2, glucose 10, N-2-hydroxyethylpiperidine-N'-2-ethanesulphonic acid (HEPES) 10; (B) experimental external solution: NaCl 150, KCl 5, $CaCl_2$ 2.6, $MgCl_2$ 1.1, glucose 10 and HEPES 10. The pH was adjusted to 7.4 by adding tris (hydroxymethyl)aminomethane (Tris-base). The internal perfusion solution (mM): KF 50, K-aspartate 50, Tris-Cl 30, $MgCl_2$ 1, ATP-$Na_2$ 1 and HEPES 10; the pH was adjusted to 7.2 with Tris-base.

### Internal perfusion by suction-pipette

Freshly dissociated VMH neurones were taken up by suction into a tapered capillary with a fire-polished tip having a diameter of approximately 500 $\mu$m and transferred into a culture dish (3 cm in diameter). Single neurones were clearly visible under an inverted microscope with magnification of 320 ×. A suction-pipette technique was used for internal perfusion (Hattori et al., 1984; Ishizuka et al., 1984; Akaike et al., 1986).

A Pyrex-glass tube with a 1.5 mm outer diameter was pulled to a shank length of 3 to 4 mm. The pipette tip was fire-polished to give an inner diameter of about 3–4 $\mu$m with fire-polishing equipment (Narishige, type MF-83). One of the dendrites was drawn into the suction-pipette with slight negative pressure (5 to 10 cmHg). This portion of the dendric membrane, was ruptured by applying square command pulses of +100 mV of 10 ms duration. Adequacy of internal perfusion with the present suction-pipette technique was evaluated by determining how close the reversal potential for the glycine-gated $Cl^-$ current ($E_{Gly}$) was to the $Cl^-$ equilibrium potential ($E_{Cl}$) of about −40 mV, calculated from the Nernst equation based on the $Cl^-$ activities estimated by a F10121Cl $Cl^-$ electrode connected to an ION85 Analyzer (Radiometer, A/S).

### Rapid drug application using 'concentration-clamp' technique

The drug-application method used was described in detail in a previous paper (Akaike et al., 1986). After setting a cell in the tip of the suction-pipette as described above, the tip was placed in a plastic tube through a circular hole with a diameter of 500 $\mu$m. The lower end of this tube can be placed in each of several dishes on a turntable which contains external solution and the required drugs. Onset and offset of quick perfusion of the solutions are operated by an electromagnetic valve, driven by 24 volts d.c. supplied by a stimulator (Nihon Koden, SEN-7103). The exchange of external solution took 2 to 4 ms for the isolated VMH neurones.

### Electrical measurement

The membrane current was measured through a Ag-AgCl wire in the suction-pipette holder. The reference electrode was also a Ag-AgCl wire in a Ringer-agar plug. The resistance between the suction-pipette filled with the internal solution and the reference electrode was about 2 M$\Omega$. Both electrodes were connected to a patch-clamp amplifier (List-Medical, EPC-7). The current and voltage were monitored on a digital storage oscilloscope (National, VP-5730A), simultaneously recorded on an ink recorder (Rikadenki, R-22) and stored on an FM data recorder (TEAC, MR-30).

### Drugs

Drugs employed in the present experiments were as follows. Collagenase (Sigma, type I), trypsin (Difco, 1: 250); α-amino acids: glycine (Gly) (Nakarai), L-α-alanine (L-α-Ala), D-α-alanine (D-α-Ala), L-serine (L-Ser), D-serine (D-Ser), L-cysteine (L-Cys) (Tokyo Kasei); β-amino acids: β-alanine (β-Ala), taurine (Tau) (Tokyo Kasei); γ-aminobutyric acid (GABA) (Tokyo Kasei); other glycine derivatives: 2-amino-isobutyric acid (2-AIBA), glycine methylester (Gly-Met), glycine ethylester (Gly-Et), oxamic acid and N-acetyl glycine (N-Ac-Gly) (Tokyo Kasei). Specific antagonists: strychnine [Sigma], bicuculline methiodide [Sigma], picrotoxin [Sigma], and 1,2,4-benzothiadiazine-1,1-dioxide (TAG). TAG was kindly donated by Dr Okamoto. Drugs were dissolved in the test solutions just before use. All experiments were carried out at room temperature (22–25°C).
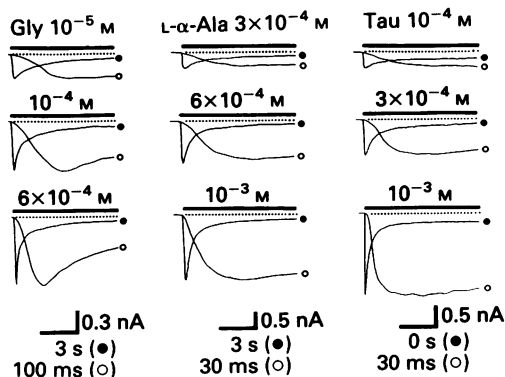
**Figure 1** Glycine (Gly)-, L-α-alanine (L-α-Ala)- and Taurine (Tau)-induced $Cl^-$ currents ($I_{Cl}$) recorded from rat isolated ventromedial hypothalamic (VMH) neurones. Holding potential ($V_H$) was −70 mV. Data were obtained from 3 different cells. Current traces were superimposed by different time scales.



**Figure 2** Dose-response curves for α- and β-amino acids at a $V_H$ of −70 mV. Abscissa scale: concentration of amino acids. Ordinate scale: relative currents induced by various amino acids normalized to the peak current induced by $10^{-4}$ M glycine alone. Solid lines for all amino acids except glycine were fitted by eye. The theoretical curve for glycine was reconstructed from equation [1] using the maximal response ($I_{max}$) of 1.7, the concentration for half $I_{max}$ ($K_a$) of $9 \times 10^{-5}$ M and the Hill coefficient (n) of 1.8. Glycine (▽, n = 8); L-α-alanine (○, n = 9); D-α-alanine (●, n = 6); L-serine (△, n = 5); D-serine (▲, n = 4); taurine (□, n = 6); β-alanine (■, n = 6). Vertical bars give s.e.mean.

## Results

Glycine, L-α-Ala, D-α-Ala, L-Ser, D-Ser, L-Cys, β-Ala, Tau, Gly-Met, Gly-Et, 2-AIBA, oxamic acid and N-Ac-Gly were applied to rat isolated VMH neurones by the 'concentration-clamp' technique. Gly, β-Ala, Tau, L-α-Ala and L-Ser produced inward $Cl^-$ currents ($I_{Cl}$) which had similar response patterns in their activation and desensitization phases (Figure 1). Both Gly-Met and Gly-Et gave much weaker responses while 2-AIBA, L-Cys, D-Ser, oxamic acid and N-Ac-Gly did not elicit any response. In Figure 1, each response is shown at superimposed fast and slow sweep speeds in order to show the activation and desensitization processes clearly.

Responses to each amino acid were normalized to the peak $I_{Cl}$ induced by $10^{-4}$ M glycine. The theoretical curve for the response to glycine was drawn according to equation [1] using the maximal response ($I_{max}$) of 1.7, the concentration for half $I_{max}$ ($K_a$) of $9 \times 10^{-5}$ M and the Hill coefficient (n) of 1.8:

$$I = I_{max} \frac{C^n}{C^n + K_a^n} \qquad [1]$$

where I is the observed drug-induced $I_{Cl}$ and C is the drug concentration. β-Ala was almost equipotent with glycine as an agonist at concentrations up to $10^{-4}$ M, although it produced smaller responses than that of glycine at concentrations over $10^{-4}$ M. Other α- and β-amino acids such as Tau, L- and D-α-Ala and L-Ser were weaker agonists than β-Ala, as shown in the dose-response curves (Figure 2). The tested amino acids, except for β-Ala, did not achieve the same $I_{max}$ as glycine, whereas the $K_a$ for glycine
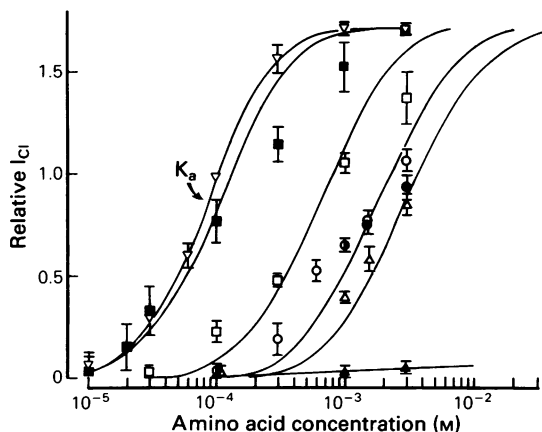
was lower than for all other amino acids. Using the differences in potency and efficacy for each amino acid, the activity of each amino acid on the glycine receptor was evaluated in the order of glycine > β-Ala > Tau > L- and D-α-Ala > L-Ser ≫ D-Ser.

Inhibitory effects of strychnine, TAG, bicuculline and picrotoxin on the responses induced by amino acids were examined. Glycine, L-α-Ala and Tau were used at concentrations nearly equal to their $K_a$ values of $10^{-4}$, $10^{-3}$ and $10^{-3}$ M, respectively. Figure 3 shows typical examples of the effects of strychnine and bicuculline on $I_{Cl}$ induced by these amino acids. Strychnine ($10^{-8}$ M) inhibited the glycine-, L-α-Ala- and Tau-induced responses by about 30 to 50% without changing either the activation or desensitization kinetics. Both bicuculline and TAG failed to inhibit these responses at low concentrations and picrotoxin gave only a very slight inhibition (less than 5%) even at concentrations greater than $10^{-5}$ M.

Effects of antagonists on $10^{-4}$ M glycine and $10^{-3}$ M Tau-induced currents were evaluated in a dose-dependent manner. The threshold concentration for an effect of strychnine on glycine and Tau-induced $I_{Cl}$ was $10^{-9}$ M, in both cases with an $IC_{50}$ value of $2 \times 10^{-8}$ M. Maximal inhibition for both
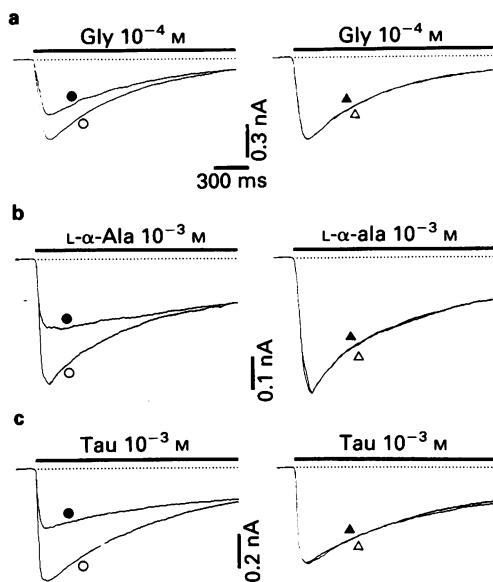
**a**



Gly $10^{-4}$ M      Gly $10^{-4}$ M

$\overline{|}$ 0.3 nA

$\overline{300\ ms}$

**b**

L-$\alpha$-Ala $10^{-3}$ M      L-$\alpha$-ala $10^{-3}$ M

$\overline{|}$ 0.1 nA

**c**

Tau $10^{-3}$ M      Tau $10^{-3}$ M

$\overline{|}$ 0.2 nA

**Figure 3** Effects of strychnine ($10^{-8}$ M at ●) and bicuculline ($10^{-5}$ M at ▲) on glycine (Gly)-, L-$\alpha$-alanine-(L-$\alpha$-Ala) and taurine (Tau)-induced $I_{Cl}$. Open symbols indicate controls. (a), (b) and (c) were obtained from 3 different neurones. $V_H$ was $-70$ mV.
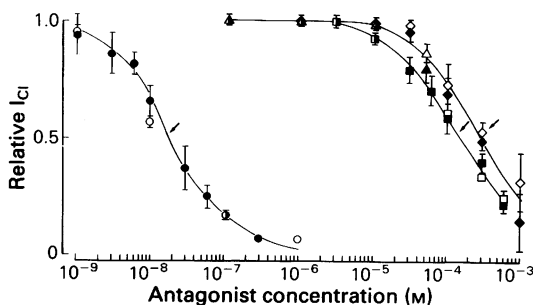


**Figure 4** Inhibition of glycine (Gly)- and taurine (Tau)-induced $I_{Cl}$ by various antagonists. Abscissa scale: concentration of antagonists. Ordinate scale: relative currents normalized to the peak current induced by $10^{-4}$ M glycine or $10^{-3}$ M taurine alone. For taurine $10^{-3}$ M: symbols indicate (○) + strychnine; (□) + picrotoxin (△) + bicuculline and (◇) + TAG(6-aminomethyl-3-methyl-4H, 1,2,4-benzothiadiazine-1, 1-dioxide hydrochloride). For glycine $10^{-4}$ M: symbols indicate (●) + strychnine; (■) + picrotoxin; (▲) + bicuculline and (◆) + TAG. Neurones were pretreated by antagonists before glycine or taurine plus one of the antagonists were applied. Arrows indicate $IC_{50}$. Each point is the average of 5 to 8 neurones and the vertical bars give s.e.mean.

responses was achieved at $10^{-6}$ M strychnine. Inhibitory effects of picrotoxin, bicuculline and TAG became evident only at a concentration of $10^{-5}$ M. The $IC_{50}$ values obtained from the inhibition curves were $1.5 \times 10^{-4}$ M for picrotoxin, $3 \times 10^{-4}$ M for bicuculline and $3 \times 10^{-4}$ M for TAG.

The cross-desensitization between $\alpha$-, $\beta$- and $\gamma$-amino acid-induced responses was examined and the interactions of glycine, L-$\alpha$-Ala, Tau and GABA are summarized in Figure 5. Figure 5a shows that pretreatment with GABA did not affect the successive glycine responses. The L-$\alpha$-Ala-induced current was unaffected by pretreatment with GABA but was completely inhibited by pretreatment with glycine (Figure 5b). The Tau-induced current was not modified by pretreatment with GABA but completely inhibited by pretreatment with glycine (Figure 5c). Cross-desensitization with glycine was also observed with responses to other $\alpha$- and $\beta$-amino acids such as D-$\alpha$-Ala, L-Ser and $\beta$-Ala (not shown).

The effects of pretreatment with $\alpha$-, $\beta$- and $\gamma$-amino acids on the glycine response were examined quantitatively. Figure 6a shows the experimental protocol. The results are summarized in Figure 6b. At concentrations up to $3 \times 10^{-3}$ M, GABA pretreatment had no effect on the successive glycine responses. On the other hand, pretreatment with other $\alpha$- and $\beta$-amino acids (L-$\alpha$-Ala, D-$\alpha$-Ala, L-Ser, Tau and $\beta$-Ala) suppressed the successive glycine responses in a dose-dependent manner. Pretreatment with D-Ser at concentrations above $10^{-3}$ M produced very slight suppression of the glycine response. The relative order of the $IC_{50}$ values for each amino acid was identical to the relative order of their $K_a$ values. The $IC_{50}$ values were approximately $2 \times 10^{-5}$ M for $\beta$-Ala, $10^{-4}$ M for Tau and $3 \times 10^{-4}$ M for L- and D-$\alpha$-Ala and L-Ser.

Cross-desensitization between glycine and various $\alpha$-, $\beta$- or $\gamma$-amino acid-induced current is shown in Figure 7. There was no cross-desensitization between glycine and GABA-induced currents. On the other hand, $\alpha$- and $\beta$-amino acid-induced currents had strong inhibitory effects on the succeeding glycine-induced current, indicating cross-desensitization.

**Discussion**

The responses induced by glycine, Tau, and other active $\alpha$- and $\beta$-amino acids were preferentially inhibited by the specific glycine receptor antagonist, strychnine (Curtis *et al.*, 1968; Graham *et al.*, 1985). On the other hand, the selective GABA$_A$ receptor antagonist, bicuculline (Curtis *et al.*, 1971; Akaike *et al.*, 1985; Yakushiji *et al.*, 1987), the Cl$^-$ channel blocker, picrotoxin (Constanti, 1978; Yakushiji *et al.*,
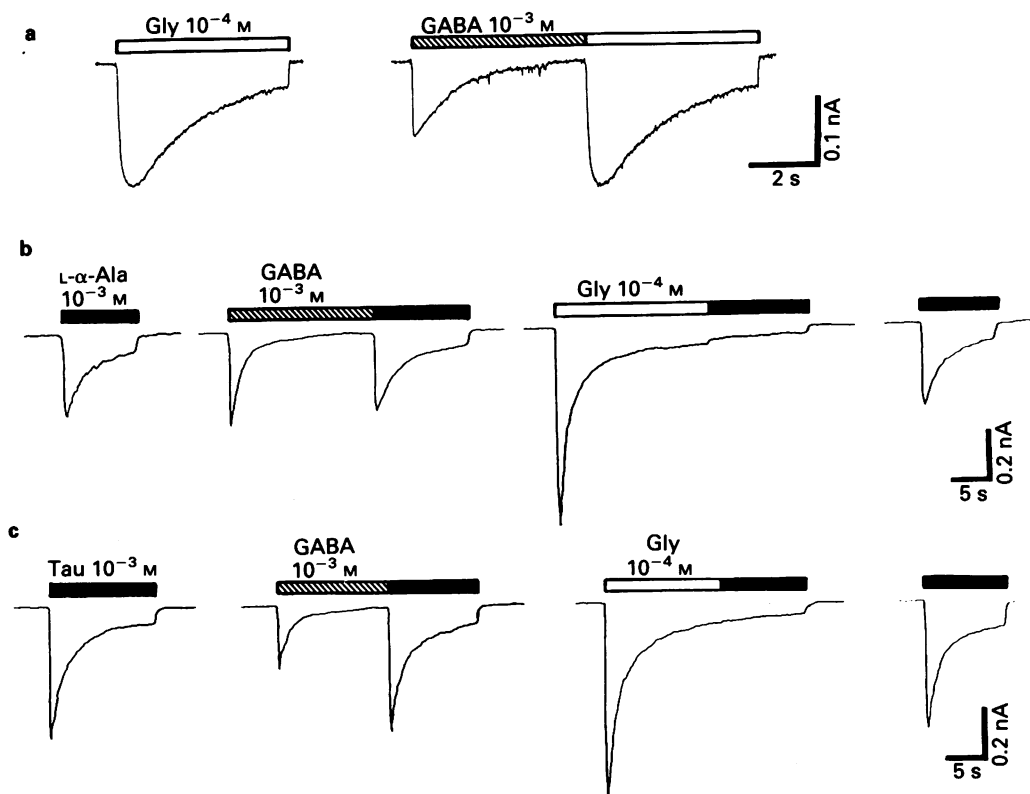
**Figure 5**  Interaction between responses induced by $\alpha$-, $\beta$- and $\gamma$-amino acids at a $V_H$ of $-70\,mV$. (a) Effect of $\gamma$-aminobutyric acid (GABA) pretreatment on glycine (Gly)-induced current. (b) Effect of GABA or glycine pretreatment on (L-$\alpha$-Ala)-induced current. (c) Effect of GABA or glycine pretreatment on taurine (Tau)-induced current. (a), (b) and (c) were obtained from different cells. Open bars: glycine treatment; hatched bars: GABA treatment; solid bars: L-$\alpha$-Ala (b) or taurine (c) treatment.

1987) and the Tau receptor antagonist, TAG (Martin et al., 1981; Yarbrough et al., 1981; Okamoto et al., 1983) failed to antagonize the $\alpha$- and $\beta$-amino acid-induced $I_{Cl}$. These results indicate that the above effects induced by glycine, Tau and the active $\alpha$- and $\beta$-amino acids are all mediated through the strychnine-sensitive glycine receptor.

On the basis of the values obtained for $I_{max}$ and the efficacy of every amino acid (Figure 2), the relative order of activity was estimated as glycine $> \beta$-Ala $>$ Tau $>$ L- and D-$\alpha$-Ala $>$ L-Ser. Ionophoretic application of glycine and related amino acids depressed the firing of spinal dorsal horn interneurones, Renshaw cells and cortical neurones of cat (Curtis et al., 1968). The potency-order was glycine $> \beta$-Ala $>$ Tau $>$ L-$\alpha$-Ala $>$ L-Ser. This similarity may also indicate that the glycine receptors in rat isolated VMH neurones are likely to be identical to the strychnine-sensitive glycine receptor in the previous reports.

In these experiments, pretreatment with GABA did not affect the successive glycine-induced response but pretreatment with glycine inhibited the successive $\alpha$- and $\beta$-amino acid-induced responses. In VMH neurones the glycine-induced responses are mediated by $Cl^-$ and there is no significant shift of $E_{Cl}$ during glycine application (Akaike & Kaneda, unpublished observation). Furthermore, the GABA-induced response is also mediated by $Cl^-$ (not shown). If the shift of $E_{Cl}$ occurs because of pretreatment with GABA, successive glycine-induced response should be smaller than the glycine-induced response without pretreatment with GABA. But as shown in Figure 5, the pretreatment with GABA did not affect the successive glycine-induced response, indicating that there is no significant shift of $E_{Cl}$ during GABA application. These results indicate that the observed inhibitory effects of $\alpha$- and $\beta$-amino acid-induced currents by pretreatment with glycine are due to cross-desensitization between

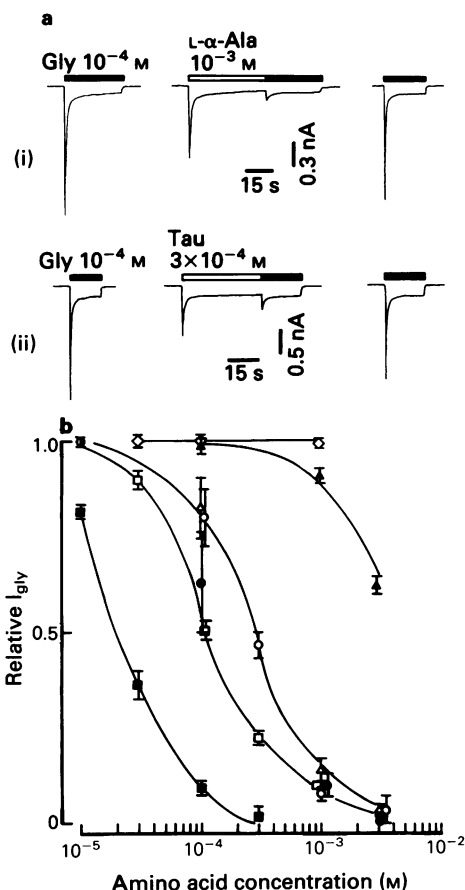**Figure 6** Effects of pretreatment with α-, β- and γ-amino acids on glycine-induced current. (a) Pretreatments with taurine (Tau) and L-α-alanine (L-α-Ala) decreased the glycine-induced current: (i) and (ii) were obtained from different cells. (b) Inhibitory effect of pretreatment with various amino acids on the glycine-induced response. Abscissa scale: concentration of amino acids during pretreatment. Ordinate scale: relative current of glycine ($I_{Gly}$) after pretreatment with various amino acids. Relative $I_{Gly}$ was normalized to the control response induced by $10^{-4}$ M glycine alone: (○) L-α-alanine; (●) D-α-alanine; (△) L-serine; (▲) D-serine; (□) taurine; (◇) GABA and (■) β-alanine. Each point is the average of 4–7 neurones and bars indicate s.e.mean.
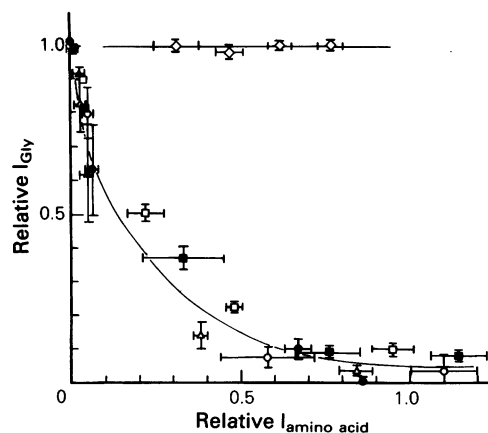


**Figure 7** Cross-desensitization between glycine- and other amino acids. Abscissa scale: relative current induced by pretreatment with amino acids normalized to the control response of $10^{-4}$ M glycine. Ordinate scale: relative $I_{Gly}$, which followed the pretreatment with various amino acids, normalized to the control response of $10^{-4}$ M glycine. Data were quoted from Figure 6b; symbols as in Figure 6b.

glycine and other amino acids. On the basis of the above results we evaluated the relationship between structure and activity of both α- and β-amino acids and several glycine derivatives on strychnine-sensitive glycine receptor. Figure 8 summarizes the structures of the various agonists used.

β-Amino acids (β-Ala and Tau) were more potent than the α-compounds in depressing the glycine response by cross-desensitization. β-Amino acids are regarded as being more potent agonists on the glycine receptor than α-amino acids except for glycine itself. As shown in Figure 8, β-amino acids are longer than the α-amino acids by a distance of one methylene group between the amino and the acidic group. It is therefore unlikely that β-amino acids act on the glycine receptor in an extended conformation. Construction of β-Ala and Tau molecules, using the C.P.K. model (space field model), can give a folded conformation with similar spatial positions for the amino group and the acidic group as those in the glycine molecule (not shown). Thus, we suggest that β-amino acids act in a folded conformation.

For the α-amino acids, including glycine, it is evident from the present study that D-Ser, L-Cys and 2-AIBA lack activity at the glycine receptor. The α-amino acids show structural variations in the functional group on the second position in the molecule. Both optical isomers of α-Ala, Ser and Cys have a methyl, methanol and methanethiol group on the second position, respectively. Glycine has no functional group on the second position, while 2-AIBA has two methyl groups (see Figure 8). Thus it is suggested that the functional group on the second position may decrease the affinity of the amino acid for the glycine receptor by steric hindrance around the second carbon in the molecule. 2-AIBA and L-Cys, which have two methyl groups and a methanethiol group, respectively, seemed to lack activity because of their large steric hindrance.

The L- and D-isomers of α-Ala were equipotent, while D-Ser was completely inactive, although L-Ser
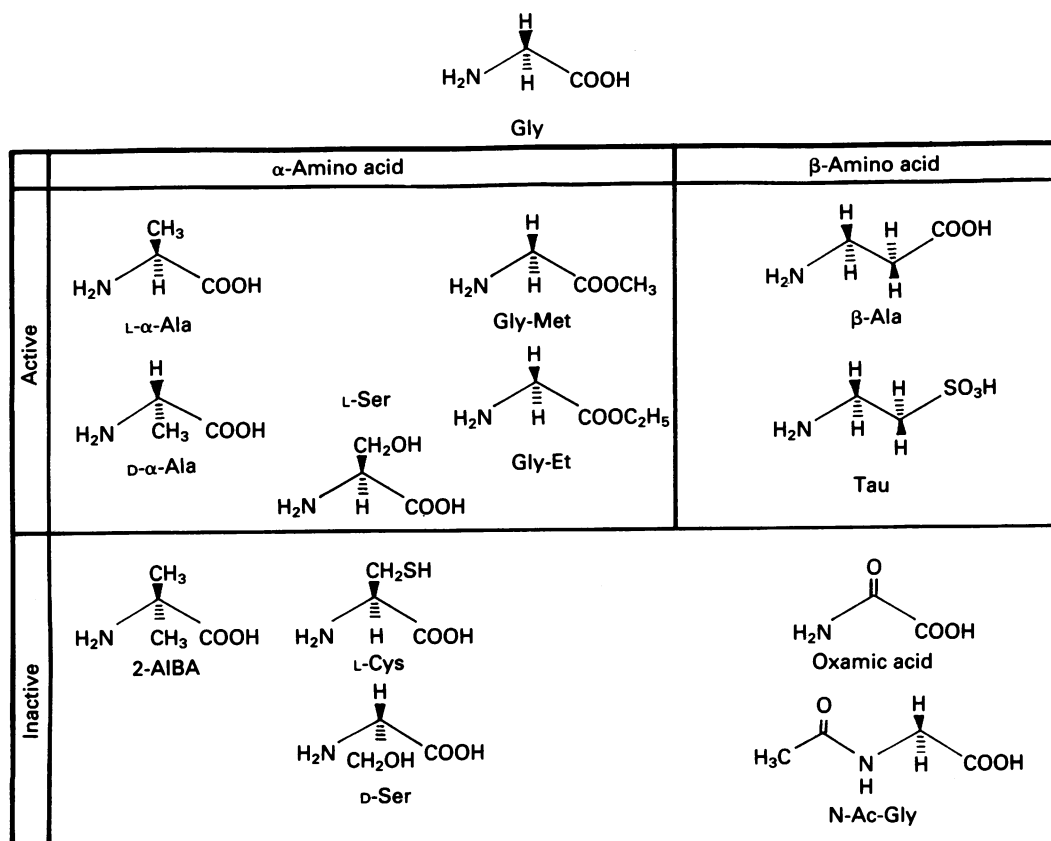
Figure 8   Chemical structures of the amino acids and their derivatives used in this study. Note the different functional group on the second carbon in different α-amino acids and the difference in molecular length between α-amino acids and β-amino acids.

was a weaker agonist. This means that the glycine receptor can discriminate between the L- and D-isomers of Ser. This discrimination involves other stereo-chemical factors, besides steric hindrance, because both optical isomers of Ser give an equivalent steric hindrance on the second C-position, analogous to L- and D-α-Ala. One of the strong modulating factors in intermolecular interactions is hydrogen bonding. Hydroxyl and thiol groups, in general, can form a hydrogen bond with carboxyl groups etc. The difference in potency between the L- and D-isomers of Ser might be attributed to hydrogen bond formation between the methanol group on the second position and the carboxyl group which probably exists in the glycine receptor protein (see Figure 8).

On the basis of the above discussion, we propose a tentative model for the specificity of the glycine receptor. The glycine receptor protein may be operated by three binding sites in its ligand-receptive zone. Two of them are thought to contribute to the

usual receptor binding by glycine and other active amino acids, attracting their amino and carboxyl groups. This would relate to receptor activation. The third site would seem to limit the binding of α-amino acids to the glycine receptor, depending on the functional group on its second position. It provides the specificity for glycine as the strongest ligand for the glycine receptor among the active amino acids which are supposed to act directly on the glycine receptor, because glycine has no functional group in the second position. In the case of Ser, the third binding site may attract the methanol group by forming a hydrogen bond. This attraction and the steric hindrance around the second carbon would make L-Ser a weaker agonist than glycine and L- and D-α-Ala. And the great difference in activity between L- and D-Ser, D-Ser being inactive although the L-isomer is a weak agonist, may indicate an asymmetric localization of the third binding site in the glycine receptor protein. It may limit the binding of D-Ser to the conventional binding site more strongly than that of

L-Ser. For Cys there is the likelihood of hydrogen bond formation and steric hindrance by the methanethiol group on the second position. Both of these stereo-chemical factors are stronger in Cys than in Ser, making even the L-isomer of Cys inactive. Relatively strong binding activity of D-Ser for the glycine receptor was reported in rat brain slices (Kishimoto *et al.*, 1981). In the present study, D-Ser failed to activate the glycine receptor in the rat isolated CNS

neurone. The relatively strong affinity of D-Ser for the glycine receptor may be related to the third binding site we propose here.

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